51752
10/593,135

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 39/395, 37/64

A1

(11) International Publication Number: WO 92/07584

(43) International Publication Date: 14 May 1992 (14.05.92)

(21) International Application Number: PCT/DK91/00317

(22) International Filing Date: 18 October 1991 (18.10.91)

(30) Priority data:
2614/90 31 October 1990 (31.10.90) DK

(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

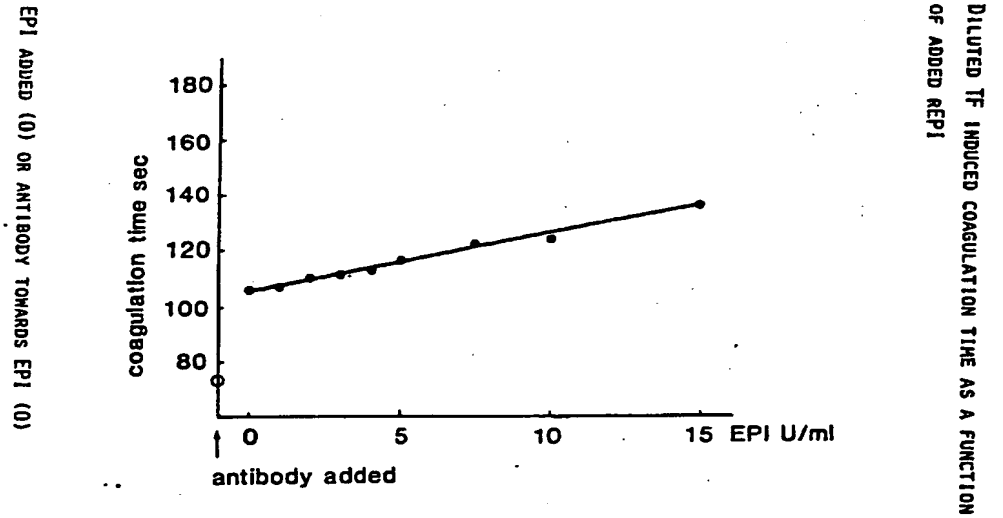
(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : NORDFANG, Ole [DK/DK]; Selskovvej 6, DK-3400 Hilleroed (DK). HEDNER, Ulla [SE/SE]; Bågängsvägen 29, S-21 620 Malmö (SE). MØLLER, Niels, Peter, Hundahl [DK/DK]; Lyngen 20A, DK-2800 Lyngby (DK).

(74) Agent: NOVO NORDISK A/S; Patent Department, Novo Allé, Dk-2880 Bagsværd (DK).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU*,TD (OAPI patent), TG (OAPI patent), US.

Published
With international search report.

(54) Title: PHARMACEUTICAL PREPARATION FOR THE TREATMENT OF PROLONGED COAGULATION TIME



(57) Abstract

A pharmaceutical preparation which, as an active component contains an agent that will block EPI activity (EPI inhibitor), is useful in the treatment of patients with prolonged coagulation time.

* See back of page

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

PHARMACEUTICAL PREPARATION FOR THE TREATMENT OF PROLONGED COAGULATION TIME

FIELD OF INVENTION

The present invention relates to agents that block EPI activity and the use of these agents to produce preparations that will affect the haemostatic balance.

BACKGROUND OF THE INVENTION

Coagulation is a complex process involving many protein factors. The coagulation process can be initiated via the intrinsic pathway through contact with a foreign surface or via the extrinsic pathway through contact with damaged tissue. By extrinsic activation the circulating blood comes in contact with tissue factor (TF). TF is a cofactor for coagulation factor VII (FVII) and the TF-FVII/TF-FVIIa complex activates FX and FIX. Due to the activation of FIX, "intrinsic coagulation" is also influenced by TF activation and an increasing amount of evidence indicate that TF-FVII activation is most important for the initiation of the coagulation reaction (Österud and Rapaport, Proc Natl. Acad Sci USA 74, p 5260, 1977). The coagulation factors of the extrinsic pathway are inhibited by EPI (Extrinsic Pathway Inhibitor) also called LACI (Broze and Miletich, Blood 69, p 150, 1987). EPI is a Kunitz type protease inhibitor which binds and inhibits FXa. The EPI-FXa complex inhibits FVIIa-TF (Rapaport, Blood 73, p 359, 1989). The significance of EPI for the coagulation reaction has not yet been established since only unphysiological high concentrations affects conventional coagulation assays (Broze et al., Biochemistry 29, p 7539, 1990). Therefore, EPI activity is measured in sophisticated assays where EPI is allowed to bind FXa and the EPI-FXa is added to TF-FVII reactants that may activate FIX or FX (Bajaj et al., J Clin Invest 79, p 1974, 1987; Sandset et al., Thromb Res 47, p 389, 1987).

In normal haemostasis the coagulation factors are in balance with each other. However, in some circumstances excessive bleeding is observed. Examples are: haemophilia caused by lack f FVIII or FIX, Idiopatic Thrombocytopenia (ITP) caused by

reduced platelet counts. Also surgery often causes excessive bleeding. Examples of agents that can be used for the prevention of bleeding are: FIX, FVIII, FVIIa, aprotinin and tranexamic acid.

DISCLOSURE OF THE INVENTION

The present invention shows that agents which block EPI acitvity will also be able to reduce bleeding tendencies. This is surprising since EPI by itself has not yet been shown to affect the haemostatic balance. However, we have made the following new observations:

1. In coagulation assay plasma EPI is more active than EPI produced by recombinant technique (rEPI).

2. Coagulation induced by very dilute TF is dependent on plasma EPI and thus EPI affects the haemostatic balance.

3. Antibodies that block EPI activity (anti-EPI) shorten the coagulation time of normal plasma.

4. Anti-EPI shortens the coagulation time of haemophilia plasma.

These observations indicate that blocking of EPI will reduce bleeding tendencies.

Previously it has only been possible to accelerate the coagulation process in normal plasma by adding a coagulation initiator (tissue factor, kaolin, etc.) or an active enzyme (thrombin, FXa, etc). Our experiments provide evidence that it is also possible to accelerate the coagulation process by blocking a plasma protein (EPI).

Intrinsic coagulation is usually measured in APTT assays where coagulation is initiated by Kaolin, phospholipids and Ca^{2+}. High concentrations of EPI are need d to affect the APTT assay (Broze, Biochemistry 29,, p 7539, 1990). Extrinsic c agulation is measured in PT assays where coagulation is initiated by tissue factor (brain extract) and Ca^{2+}. In the standard assay undiluted tissue factor is used and addition of EPI has only little effect on the coagulation times observed (see example 2). A much more physiological assay is obtained when the tissue factor solution is diluted considerably. Then the coagulation time becomes dependent on extrinsic as well as intrinsic coagulation factors (Brinkhous et al. Proc Natl Acad Sci USA 86, p 1382, 1989). Fig. 1 shows that addition of rEPI increases the coagulation time by approximately two seconds for each EPI unit added. Surprisingly addition of antibody towards EPI (removal of one EPI unit) shorten the coagulation time by 30 seconds. Control experiments were performed to ensure that the effect was really due to inhibition of EPI.

The following results were found:

A. Addition of excess antibody did not further shorten the coagulation time.

B. The titer of the antibody in EPI activity inhibition assay corresponds to the titer observed in coagulation time shortening assay.

C. The antibody was affinity purified by pure rEPI coupled to Sepharose. The resulting IgG preparation showed on a mg basis a 10 fold increase in titer towards coagulation time shortening as well as towards EPI activity.

D. When antibody was added in an amount just sufficient to shorten the coagulation time then addition of rEPI had a more pronounced effect on the coagulation time increase

compared with addition to plasma without antibody (Fig. 2).

E. EPI deficient plasma was prepared from normal plasma by immunoabsorption. This plasma had a reduced coagulation time compared with normal plasma. Addition of antibody in the coagulation analysis did not further shorten the coagulation time of the deficient plasma.

Antibodies that block EPI activity have been described in the literature (Novotny et al. Blood 72, p 2020, 1988). However, inhibition of plasma EPI by an antibody has not been described and it has not been observed that such an antibody may have a significant direct effect on coagulation and consequently on the haemostatic balance.

To measure EPI, Novotny et al. use a complicated 3 stage coagulation assay. EPI sample is first incubated with TF, FVIIa, FX and Ca^{2+} is added. Then FX is added and after 1 min. incubation FX deficient plasma and rabbit brain cephalin are added. In this assay EPI inhibits the TF activity and it has been shown that anti EPI antibody inhibits the effect of EPI.

In a one stage coagulation assay a mixture of Ca^{2+} and TF are added directly to plasma and the coagulation time is recorded. In such an assay Broze et al. (Biochemistry 29, p 7543, 1990) found it necessary to add 2.5 µg EPI/ml (50 U/ml) to obtain just 50% reduction in apparent TF activity.

Surprisingly we find that adding anti-EPI to plasma (removing 1 U of EPI) significantly affect the coagulation time when coagulation was initiated directly by dilute TF. This coagulation assay mimmics the physiological situation since it is the only type of coagulation assay that is dependent on the presence of both FVII, FVIII and FIX all factors that are necessary to obtain normal haemostasis.

Furthermore, plasma EPI has been purified (Novotny et al. J Biol Chem 264, p 18832, 1989). However, it has not been observed that the plasma EPI has a significant effect on coagulation, at least not to such a degree that the inhibition of EPI would affect the haemostatic balance. Thus it has been stressed that documentation for the psysiologic importance of EPI remains to be provided (Broze, Biochemistry 29, p 7539, 1990). Our observation, that plasma EPI significantly affects the coagulation time of plasma, provides evidence that EPI has an important role for the haemostatic balance. Furthermore, our experiments provide evidence that blocking of EPI will be able to reduce bleeding tendencies.

SUMMARY OF THE INVENTION

In its first aspect the present invention is thus related to a pharmaceutical preparation for the treatment of patients with prolonged coagulation time wherein the preparation as an active component contains an EPI inhibitor.

In a second aspect the invention relates to a method for treating patients with a prolonged coagulation time wherein a preparation, containing an EPI inhibitor, is administered to the patient.

In a third aspect the invention relates to the use of an EPI inhibitor for the production of a pharmaceutical preparation for the treatment of patients with prolonged coagulation time.

EXPERIMENTAL PART

Preparation of anti-EPI antibodies: Recombinant human EPI (rEPI) was obtained from transfected BHK cells as described by Pedersen et al. (J Biol Chem, 265, p 6786-6793, 1990). rEPI was purified by heparin affinity chromatography, ion exchange and

r versed phase chromatography (Nordfang et al., Biotech Plasma Prot p 98, 1990). rEPI obtained in this way was pure judged from SDS-PAGE. Rabbits were immunized on day 0, 14, 35 followed by 21 days intervals. Each immunization was with 0.1 mg of rEPI in adjuvans. The first immunization was with Freunds complete adjuvants while the next immunizations were with Freunds incomplete adjuvans. The antisera obtained were tested for inhibition of EPI activity in EPI activity assay and the inhibition was quantitated like FVIII inhibiting antibodies in Bethesda assay: equal volumes of diluted antiserum and EPI (1 U/ml) were incubated for 2 hours at 37°C. EPI activity was measured, and the dilution of antiserum that inhibits the activity by 50% gives the titer. The rabbit antisera had inhibiting titers between 1000 and 4000 "Bethesda-like" units/ml towards both rEPI and human plasma EPI. Below 50 fold dilution serum from unimmunized rabbits did not influence the activity of the EPI sample (1 U/ml). IgG was purified from the antisera by anion exchange chromatography. The IgG preparation with 8 mg IgG/ml contained 2000 "Bethesda-like" inhibiting units/ml towards human plasma EPI.

Assay for EPI activity: EPI was measured in a chromogenic microplate assay, modified after the method of Sandset et al., (Thromb Res 47, P 389, 1989). Heat treated plasma pool was used as a standard. This standard is set to contain 1 U/ml of EPI activity. Standards and samples were diluted in buffer A (0.05 M tris/0.1 M NaCl/0.1 M Na-citrate/0.02% NaN_3/pH 8.0) containing 2 µg/ml polybrene and 0.2% bovine serum albumin. FVIIa/TF/-FX/$CaCl_2$ combination reagent was prepared in buffer A and contained 1.6 ng/ FVIIa (Novo Nordisk A/S), human tissue factor diluted 60 fold (Hjort, Scand J Clin Lab Invest 9, 1957), 50 ng/ml FX (Sigma) and 18 mM $CaCl_2$. The assay was performed in microplate strips at 37°C. 50 µl of samples and standards were pipetted into the strips and 100 µl combination reagent was added to each well. After 10 minutes incubation, 50 µl of FX (3.2 µg/ml) was added to each well and after another 10 minutes 25 µl of chromogenic substrate for FXa (S2222) was added 10

minutes after the addition of substrate. The reaction was stopped by addition of 50 µl 1.0 M citric acid pH 3.0. The microplate was read at 405 nm.

Coagulation assay: Coagulation activity was measured using an ACL coagulation apparatus. 20 µl of antibody solution or EPI solution was incubated with 200 µl plasma for 15 minutes at room temperature. After preheating to 37°C the ACL mixes 75 µl of plasma sample with 75 µl of diluted TF in 20 mM $CaCl_2$, 50 mM NaCl, 17 mM imidazole, 33 µg/ml BSA, pH 7.4.

EXAMPLE 1

Coagulation assay was performed as described above using 20,000 fold dilution of tissue factor. Table 1 shows the effect of adding anti-EPI to 10 individual donor samples, 7 haemophilia A samples and 1 haemophilia B sample. It appears that a considerable shortening of coagulation time was observed, especially for the haemophilia samples. These data reflect the tentative greater importance of a powerfull extrinsic, FVII dependent pathway in the case of an impaired intrinsic coagulation pathway (haemophilia A and B).

Table 1. Effect of anti-EPI on the coagulation time of individual plasma samples.

	donors (n=10)	haem A (n=7)	haem B (n=1)
Coagulation time without Ab range	102 91-113	131 104-165	165
Coagulation time with anti-EPI range	77 71-87	92 82-113	116
Reduction, sec. range	26 11-39	39 22-63	49

EXAMPLE 2

Coagulation assay was performed with a normal plasma pool using different dilutions of TF. Table 2 shows that the more TF is diluted, the more significant is the effect of adding anti EPI to plasma. This illustrates that the longer the coagulation time, the more effective will anti-EPI be in shortening the coagulation time.

Table 2

Effect of adding anti-EPI or EPI as a function of coagulation time.

	Coagulation time in seconds				
Dilution of TF	Normal plasma	Normal plasma with anti-EPI	% of NP	NP with 16 U/ml of rEPI add.	%of NP
60	16.6	16.1	97	17.6	106
300	28.1	24.5	87	31.4	112
1,500	49.0	38.9	79	59.0	120
7,500	92.0	64.8	70	123	134
15,000	121	80.5	67	>165	>136
21,000	143	91.3	64	>165	>
30,000	>165	102	<62	>165	>
45,000	>165	117	<	>165	>

EXAMPLE 3

Citrate plasma was drawn from a patient after Cardiopulmonary bypass surgery (CPB). In this procedure the patient is anti-coagulated with large doses of heparin. After operation the heparin is neutralized by injection of protamine sulphate. Measured by a chromogenic FXa inhibition analysis the plasma sample contained no heparin. In Sandset EPI assay the sample contained 2 U/ml of EPI activity. Table 3 shows the dilute TF coagulation time of this plasma before and after addition of anti-EPI. It appears that the coagulation time is prolonged and that anti-EPI can shorten the coagulation time. Thus increased

EPI levels may be one of the reasons why CPB patients bleed during and after the operation.

Table 3. Effect of anti-EPI on the coagulation time of plasma from a CPB pateint.

	Donor pool plasma	CPB patient plasma
Coagulation time without antibody	100	>165
Coagulation time with anti-EPI	70	82

EXAMPLE 4

Heparin binding protein (HBP) WO89/08666 is a protein from neutrophil granula that shows high homology to elastase. HBP is not an enzyme by itself, but seems to bind enzyme inhibitors. When tested in EPI assay the titer of HBP towards EPI is 10 "Bethesda like" units/mg. HBP also blocks the inhibition of FXa by EPI with a corresponding titer. When added to normal plasma in a concentration of 150 µg/ml, the dilute TF induced coagulation time was shortened (Table 4).

Table 4. Effect of HBP on the coagulation time of normal plasma:

	Coagulation time, sec.
Normal plasma	98
Normal plasma with anti-EPI	70
Normal plasma with HBP	81

CLAIMS

1. A pharmaceutical preparation for the treatment of patients with prolonged coagulation time c h a r a c t e r i z e d in that the preparation as an active component contains an EPI inhibitor.

2. A pharmaceutical preparation according to claim 1, wherein the coagulation malfunction is due to haemophilia A or B.

3. A pharmaceutical preparation according to claim 1, wherein the coagulation malfunction is due to Idiopatic Thrombocytopenia (ITP), surgery procedures or DIC.

4. A method for treating patients with prolonged coagulation time, which comprises administering to the patient a therapeutically effective dosage of the preparation according to claim 1.

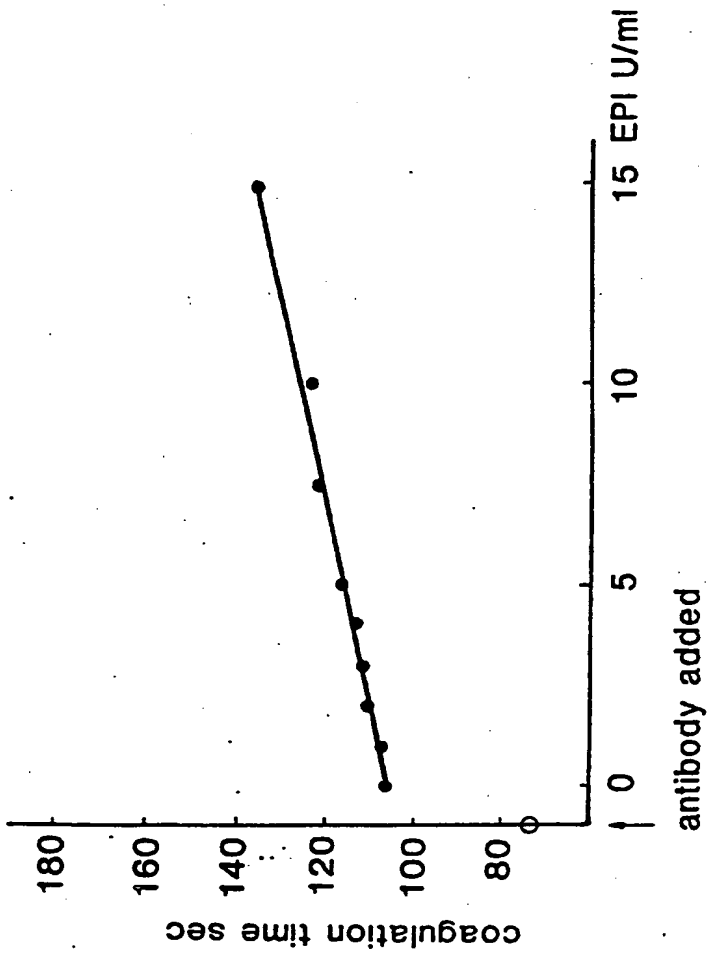
5. A method according to claim 4, wherein the coagulation malfunction is due to haemophilia A or B.

6. A method according to claim 4, wherein the coagulation malfuntion is due to Idiopatic Thrombocytopenia (ITP), surgery procedures or DIC.

7. Use of an EPI inhibitor for the production of a pharmaceutical preparation for the treatment of patients with prolonged coagulation time.

1/2

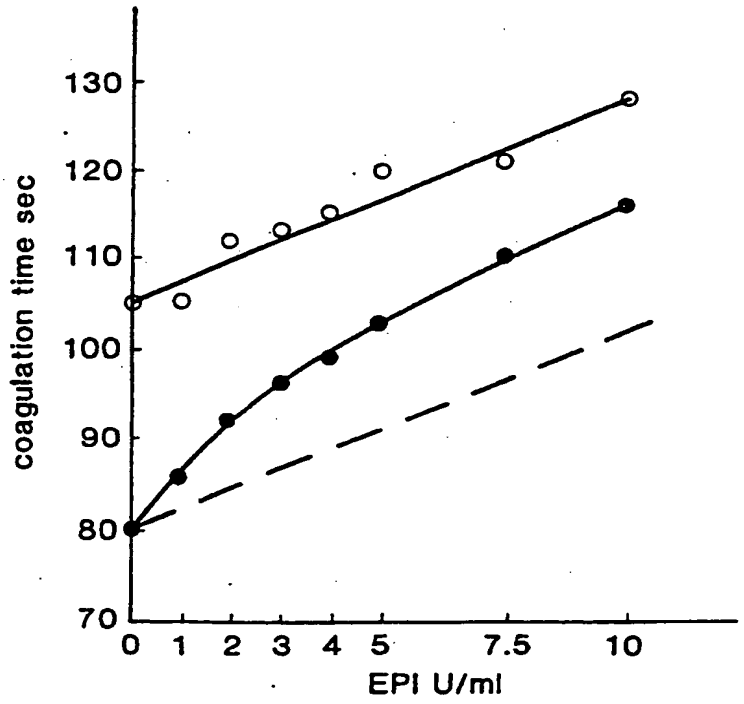
DILUTED TF INDUCED COAGULATION TIME AS A FUNCTION OF ADDED rEPI



EPI ADDED (O) OR ANTIBODY TOWARDS EPI (O)

FIG. 1

DILUTED TF INDUCED COAGULATION TIME OF PLASMA AND PLASMA WITH ANTI-EPI



REPI WAS ADDED IN DIFFERENT CONCENTRATIONS TO PLASMA. THE SAMPLES WERE INCUBATED WITH BUFFER (O) OR WITH ANTIBODY TOWARDS EPI (O)

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00317

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: A 61 K 39/395, 37/64

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,T	THROMBOSIS RESEARCH, Vol. 64, 1991 Dean J. Welsch et al: "Effect of lipoprotein-associated coagulation inhibitor(LACI)on thromboplastin-induced coagulation of normal and hemophiliac plasmas", see page 213 - page 222 especially page 220-21 1st paragraph --	1-7
P,X	THROMBOSIS RESEARCH Suppl XIV, 1991,Anne Karin Lindahl et al: "Extrinsic pathway inhibitor (EPI) and the post-heparin anticoagulant effect in tissue thromboplastin induced coagulation", see pages 39-48 --	1,4,6,7
P,X	Dialog Information Services, File 155, Medline 66-92 Dialog accession no. 07885158, Lindahl A K: "Extrinsic pathway inhibitor (EPI) released to the blood by heparin is a more powerful coagulation inhibitor than is recombinant EPI", Thromb Res Jun 15 1991, 62 (6) p 607-14 --	1,3,7

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13th February 1992	1992 -02- 14
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Carl-Olof Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	National Library of Medicine, File Medline, Accession no 86221927, Massonnet-Castel S et al: "Partial reversal of low molecular weight heparin (PK 10169) anti-Xa activity by protamine sulfate: in vitro and in vivo study during cardiac surgery with extracorporeal circulation", Haemostasis 1986; 16 (2): 139-46	1,4,6,7
X	Dialog Information Services, File 155, Medline 66-92 Dialog accession no. 06677231, Sandset PM et al: "Heparin induces release of extrinsic coagulation pathway inhibitor (EPI).", Thromb Res Jun 15 1988, 50 (6) p 803-813	1,4,6,7
A	EP, A2, 0300988 (WASHINGTON UNIVERSITY) 25 January 1989, see page 12	1
A	BIOCHEMISTRY, Vol. 29, No. 33, 1990 George J. Broze,Jr. et al: "Regulation of Coagulation by a Multivalent Kunitz-Type Inhibitor", see page 7539 - page 7546	1-7
A	BLOOD, Vol. 73, No. 2, 1989 Samuel I. Rapaport: "Inhibition of Factor VIIa/Tissue Factor-Induced Blood Coagulation: With Particular Emphasis Upon a Factor Xa-Dependent Inhibitory Mechanism", see page 359 - page 365	1-7

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 91/00317

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/11/91
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A2- 0300988	89-01-25	AU-B-	604138	90-12-06
		AU-D-	1928688	89-02-02
		JP-A-	1047799	89-02-22
		US-A-	4966852	90-10-30